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Molecular cloning and analysis of the *CRY1* gene: a yeast ribosomal protein gene

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ABSTRACT

Using cloned DNA from the vicinity of the yeast mating type locus (*MAT*) as a probe, the wild type allele of the cryptopleurine resistance gene *CRY1* has been isolated by the technique of chromosome walking and has been shown to be identical to the gene for ribosomal protein 59. A recessive *cryR1* allele has also been cloned, using the integration excision method.

The genetic distance from *MAT* to *CRY1* is 2.2 cM, while the physical distance is 21 kb, giving a ratio of about 10kb/cM for this interval. The phenotypic expression of both plasmid borne alleles of the gene can be detected *in vivo*. The use of this gene as a hybridization probe to examine RNA processing defects in the *rna 2*, *rna 3*, *rna 4*, *rna 8*, and *rna 11* mutants is also discussed.

INTRODUCTION

The rates of synthesis of ribosomal proteins are closely coordinated under a variety of growth conditions in both prokaryotes and eukaryotes (1). Ribosome biosynthesis is consequently an excellent system for examining the mechanisms used to regulate a group of functionally related genes. In *Escherichia coli*, 52 ribosomal protein (rp) genes are organized into at least 16 operons. For many of the operons, autogenous feedback regulation of transcription or translation has been demonstrated. However, not all rp operons studied fit this model perfectly, and much work remains in determining the way in which a balance is maintained among the synthesis of ribosomal proteins, and how rp gene expression is modulated in response to bacterial growth rate and amino acid starvation (reviewed in 2). Only recently, with the isolation of recombinant DNA molecules containing *Saccharomyces*, *Drosophila*, *Mus*, and *Xenopus* rp genes, have probes become available for studying the organization and expression of eukaryotic rp genes (3-8). These genes are not significantly clustered (5, 9, 10), some of them are repeated (3, 5, 11), and in yeast, many of the rp genes contain intervening sequences (5, 9, 12, 13). Preliminary experiments using cloned eukaryotic rp genes suggest that coordinate expression of eukaryotic rp

genes occurs under a variety of conditions, and is regulated via the concentration of mRNAs and/or availability for translation of rp mRNAs (14-17).

We would like to analyze the processes regulating the expression of yeast rp genes in vivo by assaying the expression of a cloned rp gene transformed into yeast. We hope to determine which sequences in or flanking the rp gene are involved in its transcription, processing, and translation, by assaying the expression of mutants of the gene generated in vitro. These experiments require that we be able to distinguish the expression of the plasmid borne rp gene from that of the chromosomal copy of that rp gene and that the expression of such a gene can be easily assayed. If these criteria are met we should be able to isolate interesting intragenic and extragenic mutations which affect rp synthesis. For these purposes we have chosen to clone a yeast antibiotic resistance gene, cry^R1, thought to encode a ribosomal protein. Resistance to cryptopleurine, an inhibitor of protein synthesis, is due to a single recessive nuclear gene, and is a readily assayable phenotype. We anticipated that expression of either the CRY^S1 allele or the cry^R1 allele from plasmids in yeast could be distinguished from that of the chromosomal copy of the gene.

The CRY1 gene was thought to code for a ribosomal protein, since cell free translation experiments using combinations of 60S and 40S ribosome subunits from cryptopleurine sensitive and resistant yeast have demonstrated that cryptopleurine resistance in vitro is a property of the 40S subunit (18). Binding studies with cryptopleurine identified a high affinity binding site on the 40S subunit (19). The CRY1 gene is tightly linked to the yeast mating type locus MAT (18, 20, Figure 1).

By using plasmids containing DNA from the vicinity of MAT as the initial probes for several rounds of overlap hybridization screening, we have isolated a fragment of DNA 21 kb centromere proximal to the mating type locus which contains the gene for rp59 and for cryptopleurine resistance. Preliminary characterizations of the structure and expression of this gene are presented.

MATERIALS AND METHODS

Strains and Plasmids: Saccharomyces cerevisiae strains DBY745 cry (MATa leu2-3 leu2-112 ura3-52 adel cry1) and DBY745 (MATa leu2-3 leu2-112 ura3-52 adel), the YEpl3 yeast SauIIIA bank (constructed by Nasmyth and Tatchell (21) and the centromere distal MAT Hind III-EcoRI subclone were

obtained from Dr. J. Haber. The rna2 (ts368), rna3 (ts125), rna4 (ts339), rna8 (ts219), and rnall (ts382) alleles used in this study were derived by Robert Last from strains obtained from the Yeast Genetic Stock Center. Temperature sensitive segregants of crosses with each of these to wild type strains, in which temperature sensitivity segregated 2:2 with respect to wild type, were used to ensure that each strain contained a single ts mutation. The yeast Sau IIIA library in YEp24, as well as the vectors YEp24, YRp17, and YIp5 were provided by Dr. D. Botstein. The yeast partial EcoRI λ phage Charon 4A library used in this study was that previously described by Woolford and Rosbash (9). The centromere proximal MAT HindIII-EcoRI subclone, DP366, was obtained from Dr. J. Hicks. A Tyl homologous probe, pPM21, was provided by Dr. M. Olson. Yeast transformants maintained on selective media were assayed for cryptopleurine phenotype by replica plating to selective media plus 1-10 μ M cryptopleurine.

Yeast Transformation: The yeast strains DB745 and DB745 cry were transformed essentially as described by Sherman *et al* (22). Transformants containing integrating vectors were tested for stability by growing isolated colonies to stationary phase in 25 ml YEED (10-12 generations) and comparing plating efficiencies on complete and selective media.

Nucleic Acid Preparation: Bacterial plasmid DNA and λ phage Charon 4A DNA were prepared as previously described (3,9). Yeast poly(A)⁺ RNA was prepared from A364A (wild type) or rna2, 3, 4, 8, or 11 ts mutant strains either grown at 23°C or shifted to 36°C one hour prior to extraction, as described by Hereford and Rosbash (23). Yeast genomic DNA was prepared by a modification of the method of Cryer *et al* (24).

Plaque and Colony Hybridization: Phage were plated, screened, grown and purified as previously described (9). The plasmid libraries were screened by the colony hybridization protocol of Gergen *et al* (25). Approximately 10,000 colonies were screened on LB+amp plates at a density of 1,000 colonies per 9 cm petri dish. Positive colonies were picked, streaked for single colonies, and rescreened to obtain pure colonies.

Gel Electrophoresis, Transfer, and Filter Hybridization of DNA and RNA: Restriction endonucleases, DNA polymerase, and T4 DNA ligase were purchased from New England BioLabs, Boehringer Mannheim and New England Nuclear and used according to their specifications. DNA restriction fragments were electrophoresed as previously described (3). DNA was transferred to nitrocellulose filters by the method of Southern (26) with the modifications that the gels were rinsed twice in 0.25M HCl for 15

minutes prior to denaturation to aid in the transfer of large fragments, and the transfers were done in 20X SSC (3.0M NaCl, 0.3M Na citrate).

RNA (total or polyA⁺) was electrophoresed on 1.5% agarose gels containing 6% formaldehyde, and 0.02M sodium 3-[N-morpholino] propanesulfonic acid, 0.005M sodium acetate, and 0.001M EDTA, pH 7.5 (1X MOPS). RNA samples were prepared to be 6% formaldehyde, 50% formamide, and 1X MOPS (27). These samples were heated to 65°C for eight minutes, 5X sample buffer (50% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol FF, and 1X MOPS) was added, and the samples were immediately loaded on the gel for electrophoresis. These gels were blotted to nitrocellulose in 20X SSPE (20 mM disodium EDTA, 0.16M NaOH, 0.2M NaH₂PO₄, 3.0M NaCl). After transferring overnight, the filters were washed in 2X SSPE, air dried, and baked at 80°C in a vacuum oven for two hours.

Both DNA and RNA filters were hybridized to DNA labeled in vitro with ³²P by nick translation (28, 29), as described by Davis et al (30). The filters were air dried and exposed as described previously.

Hybridization Selection and Cell Free Translation of RNAs: Polypeptides encoded by cloned DNAs were identified by the method of Ricciardi et al (31). Phage or plasmid DNA (20 µg) was linearized by restriction endonuclease digestion, immobilized on a 1 cm² nitrocellulose filter, and hybridized to 15 µg of polyA⁺ RNA. These RNA samples were then translated in a cell free wheat germ extract (32) containing L[³⁵S] methionine.

Gel Electrophoresis of Proteins: Radioactively labeled translation products of RNA complementary to cloned yeast DNA were analyzed by electrophoresis on SDS-polyacrylamide gels containing a 12-20% linear gradient of acrylamide (33). Translation products were identified as ribosomal proteins by their comigration with unlabeled yeast ribosomal proteins on the two-dimensional gel system described by Warner and Gorenstein (34). Yeast ribosomal proteins were isolated as described by Warner and Gorenstein (34). In some cases, the gels were stained with silver nitrate (35).

Physical and Biological Containment: Recombinant DNAs were prepared in compliance with NIH Guidelines for Recombinant DNA Research.

RESULTS

Isolation of the CRY1 and RP59 Gene

CRY1 is approximately 2.1 cM centromere proximal to MAT on chromosome III (Figure 1). Correlations of genetic and physical

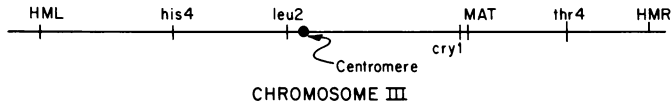


Figure 1. Genetic map of chromosome III of *Saccharomyces cerevisiae*, illustrating the relationship of MAT, CRY1, and the centromere.

distances in several intervals of the yeast genome including a large stretch of chromosome III indicated that 1 centimorgan is equivalent to ~ 3000-5000 base pairs of DNA. Thus we expected that the CRY1 gene might be 6 to 10 kb from the MAT locus, and could be isolated by chromosome walking (39, 40) using a cloned single copy DNA probe from the region around MAT. Plasmids containing HindIII-EcoRI fragments on either side of MAT were used as plaque hybridization probes to screen the recombinant phage library as described in Materials and Methods. The 0.5 kb HindIII-EcoRI fragment (DP366) was particularly useful, as it had been shown (41) to be located on the centromere proximal side of MAT, allowing us to bias our "chromosome walking" in the proper direction. The phage λ M1- λ M4 were obtained, containing overlapping fragments of yeast DNA extending 25kb centromere proximal from MAT (Figure 2). These phage were used directly as colony

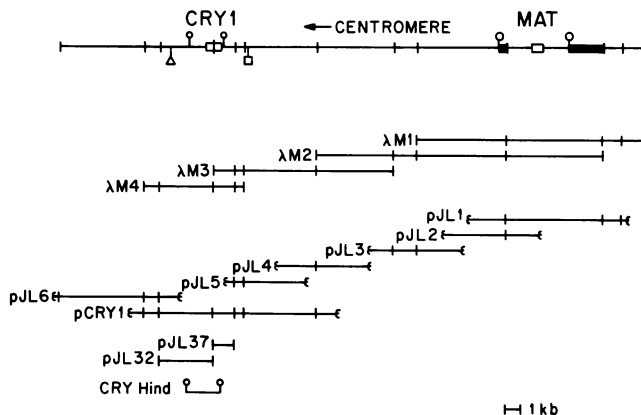


Figure 2. Cloned DNAs from the vicinity of MAT. Restriction endonuclease cleavage sites: +, EcoRI, \square , HindIII, ∇ , SalI, \circ BamHI. All EcoRI sites in the cloned interval are shown. Only the relevant cleavage sites for other enzymes are shown. The centromere proximal and centromere distal MAT HindIII-EcoRI probes used in the initial screen of the phage library are indicated by solid bars. The locations of MAT and CRY1 are shown by open bars.

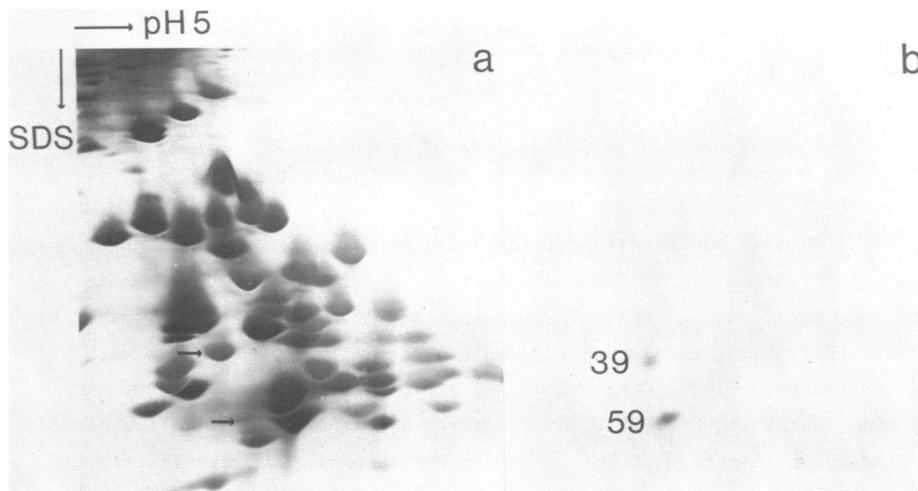


Figure 3. Two-dimensional gel electrophoresis of hybridization translation products of pJL32. a. Silver nitrate stained gel of ribosomal proteins electrophoresed as described in Materials and Methods. The positions of rp 39 and rp 59 are indicated by arrows. b. Autoradiogram of cell free translation products of RNA hybridized to pJL32. Cloned DNA containing the rp 39 gene was included in the hybridization translation as a positive control.

hybridization probes to the YEp13 and YEp24 yeast transformation plasmid libraries to isolate plasmids pJL1 - pJL6 (in YEp13) and pCRY1 (in YEp24), containing overlapping yeast genomic fragments from the same interval (Figure 2). DBY745cry yeast transformed with pCRY1 but not pJL1 - pJL6, YEp13, or YEp24 were cryptopleurine sensitive, from which we conclude that the CRY^S1 gene and sequences sufficient for its expression are present entirely within pCRY1 DNA. The CRY^S1 gene was further localized by subcloning the 2.2 kb HindIII restriction fragment of pCRY1 into YEp13. This plasmid was capable of transforming DBY745cry to leucine prototrophy and cryptopleurine sensitivity, demonstrating that this restriction fragment contains a functional copy of CRY^S1.

In order to determine whether any ribosomal protein genes were located within the cloned interval, RNA complementary to each recombinant was selected by hybridization to filter bound DNA and was translated in a wheatgerm cell free lysate. The radioactive translation products were analyzed by two dimensional polyacrylamide gel electrophoresis. As shown in Figure 3, the subclone pJL32 hybridizes to mRNA coding for a 17,000

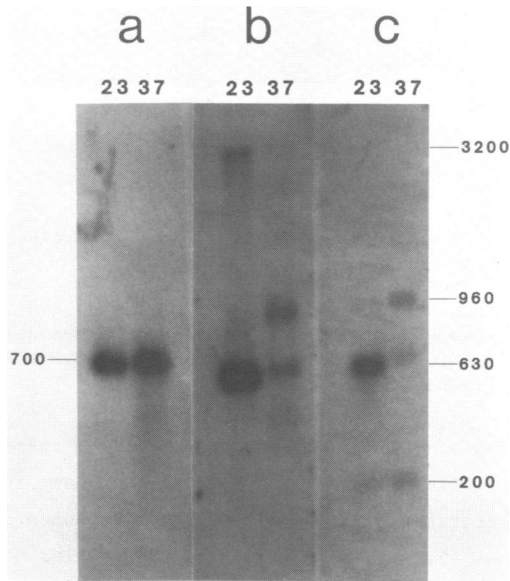


Figure 4. RNA blot analysis of transcripts in the vicinity of CRY1. PolyA⁺ RNA (2μg) from rna2 yeast grown at 23°C, or grown at 23°C and shifted to 37°C for one hour, was electrophoresed and transferred to nitrocellulose as described in Materials and Methods. The blot was probed with cloned DNA labeled in vitro with ³²P. The indicated sizes of the transcripts in nucleotides were obtained by comparison with pBR322 restriction fragments denatured as described for RNA and electrophoresed on the same gel. The probes were: (a) 1078, a plasmid containing the rp 39 gene, (b) pJL32, (c) pJL37.

dalton polypeptide which comigrates with rp59 on a two dimensional gel. Translatable rp59 mRNA was also selected by hybridization to the subclone pJL37 (data not shown) indicating that the rp59 gene spans the chromosomal Eco R1 site common to these two DNAs. This Eco R1 site is internal to the 2.2 kb HindIII restriction fragment containing CRY1 (See Figures 2 and 5).

An attempt was made to determine whether the cry^{R1} allele encoded an electrophoretic variant of rp59. However, the cell free translation products of rp59 mRNA selected from both DBY745 and DBY745cry RNA comigrated on two-dimensional gels (data not shown).

The CRY1 Gene and rp59 Gene are Identical

Evidence suggesting the identity of the CRY1 and rp59 genes was obtained by mapping transcripts in this region of chromosome III. We took advantage of the fact that the rp59 gene, like most other yeast rp genes, is known to be affected by mutant alleles of the RNA2 gene (42, and J. Larkin,

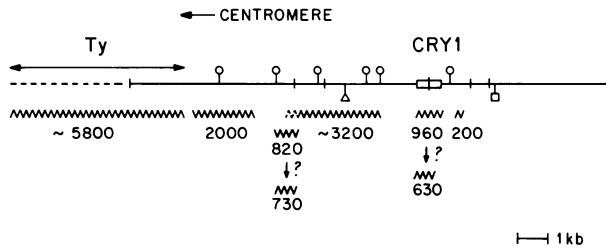


Figure 5. Locations of transcripts in the vicinity of *CRY1*. The results of the RNA blots in Figure 4 and similar experiments are summarized in this figure. Possible precursor-mature transcript relationships are indicated by arrows. The symbols for restriction endonuclease cleavage sites are identical to those in Figure 2.

unpublished). Yeast containing the temperature-sensitive *ts368* allele of *RNA2* are unable to correctly process the transcripts of many *rp* genes at the nonpermissive temperature, apparently due to the presence of intervening sequences in these genes (5,9,12). As a result, RNA species complementary to these genes which are larger than their mature mRNAs accumulate in this mutant at the nonpermissive temperature. PolyA⁺ RNA isolated from *rna2* yeast grown at 23°C (the permissive temperature) and from the same strain grown at 23°C and shifted to 37°C for one hour, were separated by gel electrophoresis under denaturing conditions, blotted to nitrocellulose, and probed with radioactively labelled DNA fragments. The results of this experiment are shown in Figure 4, and the conclusions of this and other RNA blotting experiments are diagrammed in Figure 5. As shown in Figure 4, lanes B and C, there is one transcript present at 23°C that is 630 nucleotides long, is of comparable abundance to the *rp39* mRNA (lane A), and hybridizes to both pJL32 and pJL37. The concentration of this transcript is decreased approximately tenfold in 37°C *rna2* RNA. Another transcript 960 nucleotides long hybridizes to both subclones, and its concentration is greatly increased in RNA from cells shifted to 37°C.

Thus the location and response to *rna2* of the 630 nucleotide and the 960 nucleotide transcripts suggest that they are the mature message and an incompletely processed precursor of the *rp59* gene. Northern blotting using the 2.2 kb *Hind*III restriction fragment demonstrates that these two transcripts are the only detectable transcripts complementary to this fragment (see Figure 8) which includes all of the functional *CRY1* gene. It follows that the *rp59* gene and the *CRY1* gene are identical.

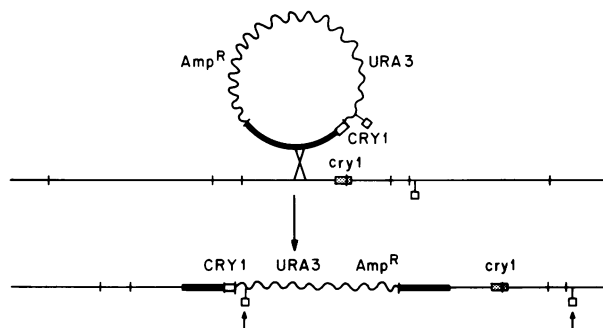


Figure 6. Cloning of the cry^{R}_1 resistance allele. The strain DBY745cry was transformed to uracil prototrophy with pJL38, a derivative of YIp5 which contains a 3.5 kb *Eco*RI restriction fragment including the centromere distal portion of *CRY1*. The expected integration event, confirmed by Southern blotting, is depicted above. Genomic DNA was isolated from a transformant, cut with *Bam*HI (arrows), ligated with T4 DNA ligase, and used to transform *E. coli* to ampicillin resistance.

Cloning the Resistance Allele of *CRY1*

For many types of genetic studies, it would be useful to have a cloned copy of the cryptopleurine resistant cry^{R}_1 allele. For this reason, the cry^{R}_1 allele was acquired by the integration-excision method (43). The 3.5 kb *Eco*RI fragment from pJL32, which contains a centromere proximal portion of the *CRY*^S₁ gene, was transferred to the integrative transformation vector YIp5. This plasmid was used to transform DBY745cry to URA^+ . Stable URA^+ transformants were obtained after 10-12 generations of nonselective growth in liquid YEPD, presumably resulting from homologous recombination of the YIp5 recombinant plasmid into the chromosomal *CRY1* locus. The expected integration event is illustrated in Figure 6. Transformants with the proper integrated structure were recognized by Southern blots to restriction fragments of genomic DNAs cut with appropriate restriction enzymes. If recombination between the plasmid and the chromosome occurs to the left of the cry^{R}_1 mutant lesion as depicted in Figure 6, then it should be possible to recover the cry^{R}_1 allele on a *Bam*HI fragment containing the ampicillin resistance gene and an *E. coli* origin of replication. Recombination to the left of cry^{R}_1 should be favored by the location of the *CRY*^S₁ sequences on the extreme right of the plasmid insert. Also, if it is assumed that the partial *CRY*^S₁ gene present on the plasmid is not functional, then only the recoverable allele of the gene should be expressed. Consistent with this expectation, all stable transformants were cryptopleurine resistant.

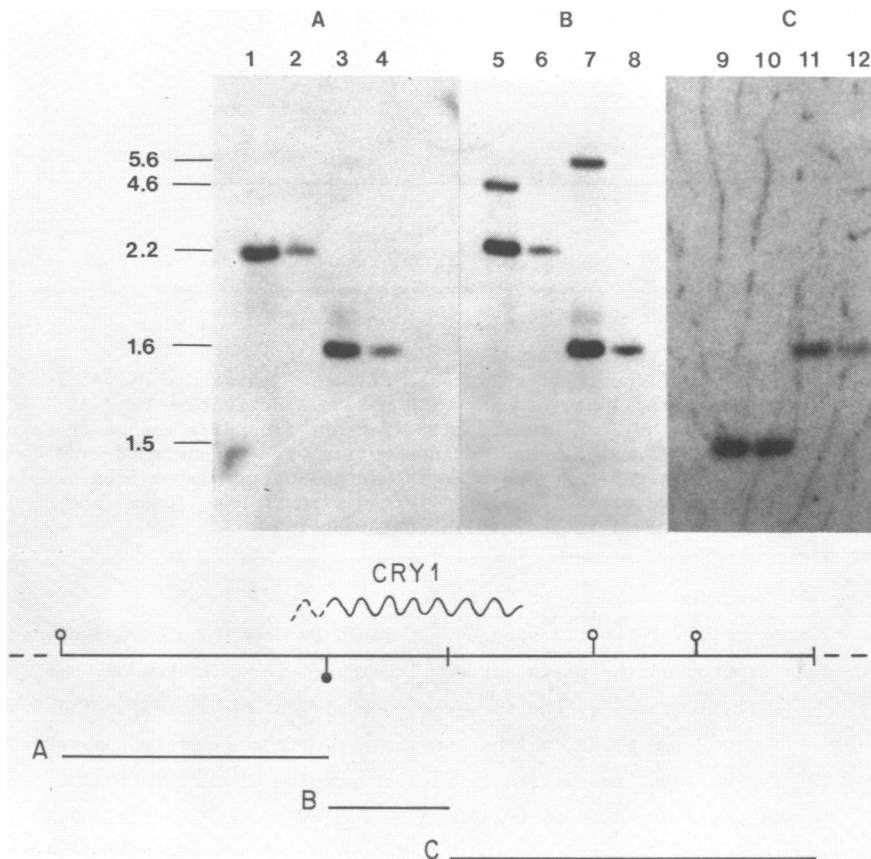


Figure 7. Is *CRY1* single copy? 5 μ g of DBY745cry genomic DNA, and approximately one genome equivalent of λ M4 DNA (see Figure 2) was digested with *EcoRI* or *HindIII*, electrophoresed and transferred as described in Materials and Methods. Blots were probed with DNA labeled *in vitro* with 32 P. Lanes 1, 3, 5, 7, 9, and 11 are genomic DNA. Lanes 2, 4, 6, 8, 10, and 12 are λ M4 DNA. Lanes 1, 2, 5, 6, 9, and 10 were digested with *EcoRI*. Lanes 3, 4, 7, 8, 11, and 12 were digested with *HindIII*. Lanes 1, 2, 3, and 4 were probed with a *HindIII*-*BglII* fragment centromere proximal to the bulk of the *CRY1* gene. Lanes 5, 6, 7, and 8 were probed with a *BglII*-*EcoRI* fragment which includes part of *CRY1*. Lanes 9, 10, 11, and 12 were probed with pJL37, which includes the centromere distal end of *CRY1*. Restriction endonuclease cleavage sites are as above except ∇ , *BglII*. Two small *HindIII* fragments predicted to hybridize to pJL37 were too small to be retained on this gel.

Genomic DNA from appropriate transformants was digested with *BamHI*, ligated under dilute conditions, and used to transform *E. coli* to ampicillin resistance. Plasmid DNA was isolated from a number of transformants, and

several of these DNAs were found to contain the expected restriction pattern. DNA containing the putative cry^R1 allele was subcloned from one of these plasmids into YEp24. When this subclone was used to transform the Cry^S strain DBY745, all of the transformants were Cry^R (see Table I and below), demonstrating the successful isolation of the cry^R1 allele from DBY745 cry.

Is the CRY1 Gene Single Copy?

Most, if not all rp genes in mammals are repeated (16). Recently, it has been suggested that some rp genes in yeast are also repeated (5). When the 2.2 kb HindIII fragment, which contains CRY1, and no other detectable genes (see Figure 5), is radioactively labeled and hybridized to Southern blots of genomic EcoRI and HindIII fragments, a single additional band was seen in each lane which was not predicted by the restriction map of the CRY1 region (Figure 7). Thus there is a sequence within the 2.2 kb HindIII fragment which is duplicated. To determine whether the duplicated sequence lies within the CRY1 gene, it was localized more specifically. A 1.1 kb HindIII-BglII fragment centromere proximal to most of the CRY1 gene (probe A, Figure 7), a 0.5 kb BglII-EcoRI fragment containing the centromere proximal portion of CRY1 (probe B, Figure 7) and pJL37 (probe C, Figure 7) were used to probe blots of genomic EcoRI and HindIII digests, as described above. The additional EcoRI and HindIII fragments (4.6 kb and 5.4 kb respectively) were homologous to the 0.5 kb BglII-EcoRI probe, but not to the HindIII-BglII fragment or to pJL37. Since the CRY1 gene extends into the EcoRI fragment cloned in pJL37, that portion of the gene within pJL37 must not be duplicated, i.e. there is only one intact CRY1 gene in Saccharomyces cerevisiae, consistent with the existence of only one genetic locus capable of conferring a high level of resistance to cryptopleurine.

Effect of rna Mutations on the CRY1 Gene

The synthesis of translatable mRNA for most ribosomal proteins is dramatically reduced in yeast containing temperature-sensitive lethal mutations in ten different genes, RNA2-RNA11. This effect has been studied in greatest detail for the ts368 allele of RNA2 and the rp51 gene, where it has been demonstrated that processing of an intervening sequence from the rp51 transcript is blocked at the nonpermissive temperature resulting in a decrease in concentration of mature rp51 mRNA and the accumulation of a higher molecular weight precursor (12). We assume that the CRY1 gene, like

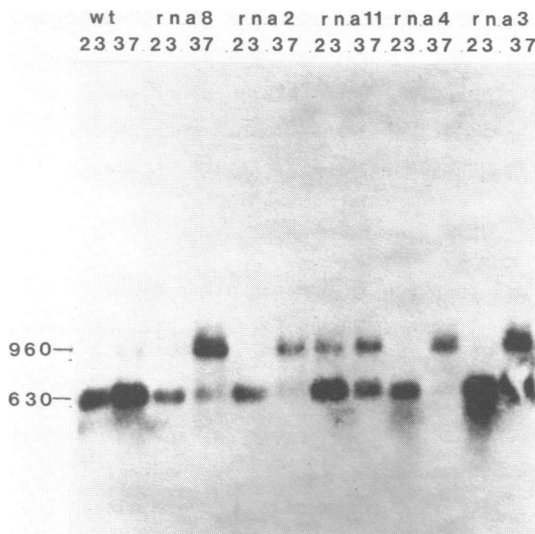


Figure 8. Molecular phenotype of *rna* mutants. Total RNA (4 μ g) from *rna2*, *3*, *4*, *8*, or *11* yeast grown at 23°C, or grown at 23°C and shifted to 37°C for one hour, was electrophoresed and transferred to nitrocellulose as described in Materials and Methods. The blot was probed with a 32 P labeled 2.2 kb *Hind*III restriction fragment containing the *CRY1* gene.

most other yeast ribosomal protein genes, contains one or more intervening sequences, since Northern blotting demonstrated that the "mature" 630 nucleotide *CRY1* transcript is decreased in concentration at 37°C in *ts368*, and a 960 nucleotide transcript homologous to the gene is increased in concentration (Figure 4, lanes B and C). We would like to study the molecular phenotype of each of the *rna* mutants, in order to learn whether mutations in each of the *RNA* genes affect processing of yeast transcripts, and, if so, what role each of the *RNA* gene products might play in the process. Cloned ribosomal protein genes provide a convenient probe to study this process. PolyA⁺ RNA was isolated from wild type (A364A), *rna2* (*ts362*), *rna3* (*ts125*), *rna4* (*ts339*), *rna8* (*ts219*), and *rna11* (*ts382*) cells grown at 23°C and shifted to 37°C for one hour prior to harvesting. These RNAs were electrophoresed on denaturing gels, blotted, and hybridized with the radioactively labeled 2.2 kb *Hind*III fragment containing the *CRY1* gene. As shown in Figure 8, the concentration of the 630 nucleotide transcript is decreased in each mutant grown at 37°C, while in each case the 960 nucleotide transcript is increased in concentration, although to variable extents in each mutant.

TABLE I

Plasmid	Phenotype			
	Transformed into DBY745		Transformed into DBY745cry	
	<u>Cry^S</u>	<u>Cry^R</u>	<u>Cry^S</u>	<u>Cry^R</u>
YEp13	20/20	0/20	0/20	20/20
YEp24	20/20	0/20	0/20	20/20
YRp17	20/20	0/20	0/20	20/20
YEp13CRY ^S ₁	N.D.		40/40	0/40
YEp24CRY ^S ₁	N.D.		24/24	0/24
YEp17CRY ^S ₁	N.D.		8/8	0/8
YIp5CRY ^S ₁	N.D.		3/6	3/6
YEp24cry ^R ₁	0/32	32/32	0/8	8/8
YRp17cry ^R ₁	31/32	1/32	0/8	8/8
YIp5cry ^R ₁	6/8	2/8	0/8	8/8

The plasmid YEp13CRY^S₁ contains the 2.2kb Hind III fragment. All other Cry plasmids contain a 5.0 Kb BamHI - SalI fragment carrying either the CRY₁ allele or the cry₁ allele. Cryptopleurine resistance was determined on plates containing 5 μ m cryptopleurine after three days of growth. Under these conditions, diploid strains heterozygous for CRY₁ were equally as sensitive to cryptopleurine as DBY745, and all transformants scored as Cry^R were fully as resistant as DBY745cry.

Expression of Either Allele of Plasmid Borne CRY₁ Can be Distinguished in Yeast

We have cloned the CRY^S₁ gene in order to obtain a yeast ribosomal protein gene which we can reintroduce into yeast by transformation, and whose expression in vivo can then be readily assayed and distinguished from that of the chromosomal copy of the gene. For this reason, the in vivo expression of both the CRY₁ allele and the cry^R₁ allele in several yeast transformation vectors was examined. The 5.0 kb BamHI-SalI fragment containing CRY^S₁ was subcloned from pCRY1 into the vectors YIp5, YRp17, and YEp24, and transformed into the Cry^R strain DBY745cry. All cells transformed to uracil prototrophy with the independently replicating, multicopy plasmid derivatives of YEp24 and YRp17 were cryptopleurine sensitive, while half of those transformed with the YIp5 derivative containing CRY^S₁ were sensitive. A similar inconsistency in phenotypes of integrative transformants has been observed at the suf2 locus, and may be attributable to gene conversion associated with the integration event (44).

Similarly, the 5.0 kb BamHI-Sal I fragment containing the cry^R₁ resistance allele was subcloned into YIp5, YRp17, and YEp24, and each recombinant was used to transform both the Cry^R and Cry^S strains DBY745cry and DBY745 to uracil prototrophy. In each case all of the DBY745cry URA⁺ transformants were CRY^R (Table I). When the cry^R₁ allele was transformed into the sensitive strain, DB745, only those yeast transformed with the more stable, high copy number plasmid derivative of YEp24 were uniformly resistant (Table I). When only one intact copy of the resistance gene cloned in YIp5 was integrated at the sensitive locus (as assayed by Southern blotting) or when the resistance allele were transformed into the sensitive strain using derivatives of the less stable plasmid YRp17, most of the URA⁺ transformants were sensitive. These results are consistent with the transforming plasmids containing the Cry^R allele of cryptopleurine, which is recessive to the Cry^S allele when one copy of each gene is present. The in vivo expression of either plasmid borne allele of CRY1 can be detected phenotypically, providing us with a variety of possibilities for assaying rp59 gene expression.

DISCUSSION

The results presented here demonstrate the isolation of the CRY^S₁ gene and its identity with the rp59 gene. CRY^S₁ was cloned by using previously cloned single copy DNA centromere proximal to the mating type locus as a probe for overlap hybridization to "walk" from MAT to CRY^S₁ on chromosome III. Cloned DNA in yeast transformation vectors was assayed for the presence of the CRY^S₁ gene by screening the plasmids for the presence of sequences capable of transforming a cryptopleurine resistant yeast strain to sensitivity. By these means we isolated the CRY^S₁ gene and localized it and sequences sufficient for its expression within a 2.2 kb HindIII restriction fragment 21 kb centromere proximal to MAT. The cry^R₁ allele of DBY745cry was cloned by the integration-excision method pioneered by Roeder and Fink (43). Hybridization translation assays and Northern blotting demonstrated that the rp59 gene is present in this 2.2 kb HindIII fragment, and that it is the only detectable gene in the fragment, from which we conclude that the CRY1 and rp59 genes are identical.

An unexpected finding was that the MAT-CRY1 physical distance was 21 kb. In most yeast strains, the genetic map distance of this interval is slightly over 2 cM (J. Haber, personal communication), giving a ratio of about 10 kb/cM. This ratio is almost double the highest previously

determined ratio of 5.6 kb/cM for the CYC1-sup4 interval on chromosome X. By taking advantage of a circular chromosome III generated via recombination between HML and MAT, Strathern *et al* (36) were able to estimate a ratio of 2.7 kb/cM for a large portion of chromosome III including the CRY1-MAT interval. This region of chromosome III shows no evidence of gross rearrangement in several strains of yeast which have been tested (this paper, and unpublished results). Studies are underway to determine the significance of this abnormally low recombination frequency and its relationship, if any, to specific sequences around MAT.

No known genes have been mapped between MAT, CRY1, and the centromere proximal gene PET18. We have detected at least eight transcripts in the 11kb immediately surrounding CRY1. One of these transcripts appears to be associated with a repeated Tyl-like element centromere proximal to CRY1. (See Figure 5, J. Larkin, unpublished data.) A small transcript homologous to sequences centromere proximal of CRY1 accumulates a potential precursor in rna2 yeast at the nonpermissive temperature (Figure 5, unpublished data). However, the *in vitro* translation products of hybrid selected RNA from this region do not comigrate with any basic or acidic ribosomal proteins on two dimensional polyacrylamide gels. Thus we do not believe that this gene codes for a ribosomal protein. We have found no other ribosomal protein genes nearby to CRY1. A similar lack of clustering of ribosomal protein genes has been found adjacent to other cloned ribosomal protein genes, consistent with the notion that coordinate expression of yeast ribosomal protein genes is not mediated by their organization into operons or tightly linked domains of chromatin.

Recently, two other antibiotic resistance genes, the trichodermin resistance gene TCM1, and the cycloheximide resistance gene CYH2, have been cloned from yeast and shown to encode ribosomal proteins L3 and L29 respectively (4,6). These two genes, as well as CRY1, have each been mapped to a single genetic locus. As expected from these results, both TCM1 and CYH1 are single copy yeast genes (4, 6). Our data suggest that there is only one complete CRY1 gene. A duplicated sequence lies centromere proximal to the EcoRI site internal of the CRY1 gene, but our data lack the resolution to determine whether this sequence lies within the CRY1 gene or is adjacent to it. Should the duplicated sequence prove to be present within the gene, at least three possibilities would be consistent with the genetics of CRY1: 1) The CRY1 gene is duplicated but the second copy of the CRY1 gene is non-functional (a pseudogene); 2) the second

sequence may have only partial homology to the CRY1 gene, and may code for a protein, not rp 59, which does not interact with cryptopleurine; 3) the duplicated sequences may be in a nontranslated portion of the gene. We note that only the 630 nucleotide and the 960 nucleotide transcripts are detected by RNA blotting using either a single copy DNA probe from CRY1, or a probe containing CRY1 sequences and the duplicated sequence.

Our data suggest that the CRY1 gene contains one or more intervening sequences totaling approximately 330 nucleotides in length which are removed via splicing from a 960 nucleotide poly(A)⁺ precursor to produce a 630 nucleotide poly(A)⁺ mRNA. As shown in Figure 8, this precursor transcript accumulates to variable extents at the nonpermissive temperature in each of the temperature-sensitive mutants rna2, rna3, rna4, rna8, and rna11. A small amount of precursor is present in wild type cells at 23°C and 37°C. Similar results have been obtained using other cloned ribosomal protein genes (Woolford, unpublished). The CRY1 gene could be a useful probe for the investigation of the processing of yeast transcripts and the role of RNA gene products in this process. That both the cry^R₁ and CRY^S₁ alleles are expressed from plasmids means that the intervening sequences of these genes are properly processed. We therefore are provided with not only a probe for RNA processing but also a template for biochemically and genetically assaying functions necessary for processing in yeast.

The ability to return DNA sequences which have been altered in vitro to living yeast cells by transformation is a powerful tool in yeast molecular biology. In preparation for the use of this approach to the study of the regulation of the CRY1 gene, we have begun to examine the expression of both unaltered alleles of this gene when introduced into yeast on vectors which have different copy numbers per cell. We have found that under appropriate conditions, the expression of either allele present on a plasmid in yeast can be distinguished. Although cryptopleurine sensitivity is dominant to resistance in heterozygous diploids or merodiploids, multiple copies of the cry^R₁ allele in a cell containing the CRY^S₁ gene on the chromosome renders the cell phenotypically resistant (Table I). This might result from an excess of "resistant" proteins being synthesized relative to "sensitive" proteins, assuming there is no bias toward assembly of either protein into otherwise functional ribosomes.

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¹Abbreviations Used

rp	ribosomal protein
kb	kilobase
SDS	sodium dodecyl sulfate
ts	temperature sensitive

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